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Monoclonal antibodies to novel myeloid antigens reveal human neutrophil heterogeneity

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ABSTRACT **Three cytotoxic murine monoclonal antibodies that recognize myeloid-specific antigens have been produced by immunization with normal human neutrophils or myeloblasts from a patient with acute myelomonocytic leukemia. Two of these, PMN 6 and PMN 29, are specific for neutrophils; the third monoclonal antibody, AML-2-23, is reactive with the majority of normal monocytes as well as a subpopulation of mature neutrophils. Although neutrophils from all individuals tested expressed these antigens, cytofluorographic analysis revealed that the percentage of cells bearing the PMN 6 and AML-2-23 antigens varied among individuals. Significant additional heterogeneity in the density of each antigen among antigen-bearing cells was also observed. All three antibodies efficiently mediated complement-dependent cytotoxicity of acute myelocytic leukemia cells yet were unreactive with lymphocytic leukemia cells. Neutrophil cytotoxicity was mediated by PMN 6 and PMN 29 but not by AML-2-23. On the other hand, AML-2-23, but not PMN 6 or PMN 29, was cytotoxic for normal monocytes and macrophages. These monoclonal antibodies may be of value in the study of normal neutrophil function and differentiation and may have clinical utility in diagnosis and therapy of myeloid leukemia.**

Neutrophilic granulocytes in the peripheral circulation appear to be a morphologically homogenous, short-lived, fully differentiated group of cells. These cells have a significant role in nonspecific antibody- and complement-directed host defense. Various correlates of neutrophil function have been identified and used to monitor the activity of these cells and their abnormalities (1–4). Although *in vitro* induction of neutrophil activity appears to mimic *in vivo* stimulation, different stimuli often induce the same response, suggesting that a common pathway may be activated through different cell surface receptors. Alternatively, or in addition, different functional activities might be associated with different subpopulations of neutrophils. In fact, there is some evidence of functionally distinct neutrophil subpopulations (5, 6).

A number of monoclonal antibodies reactive to antigens associated with human myeloid cells have recently been reported, which may be useful in dissecting neutrophil biological activities. Several of these antibodies react with antigens associated with both neutrophils and monocytes (7–9). Certain other monoclonal antibodies are specific for and react with the entire circulating neutrophil population (NCD 1, 1/12/13) (4, 10). One of these antibodies, NCD 1, as well as one whose specificity has not been defined (NCD 3), reacts with neutrophils and inhibits some but not all neutrophil functional correlates (4, 11). NCD 1 blocks lysozomal enzyme release and chemotaxis, whereas NCD 3 only inhibits *N*-formylmethionylleucylphenylalanine-induced chemotaxis. The unique specificities of these monoclonal antibodies suggest that there may be a large number of antigens that are characteristic of myeloid cells.

In the present report we describe the reactivities of three different myeloid-specific monoclonal antibodies—PMN 6, PMN 29, and AML-2-23—which appear to be useful in studies of neutrophils, neutrophil subpopulations, and myeloid leukemias.

MATERIALS AND METHODS

Production of Hybridomas. Hybrid cells making monoclonal antibodies to human neutrophils were prepared as described, with neutrophils from normal donors or blast cells from a patient with acute myelomonocytic leukemia used as immunogens (12, 13). Clones PMN 6, PMN 29, and AML-2-23, derived from two different fusions, were selected for study on the basis of their specific reactivity to neutrophils. Immunoglobulin class and subclass of each monoclonal antibody were determined as described (12).

Cells. Neutrophils used for immunizations and screenings were isolated from the peripheral blood of normal donors. Mononuclear cells were separated from neutrophils and erythrocytes (RBC) by Ficoll/Hypaque gradient centrifugation (14). RBC were separated from neutrophils by sedimentation in 2% dextran and lysis by osmotic shock. Monocytes used for screening were prepared from the mononuclear fraction by adherence to plastic Petri dishes pretreated with autologous serum for 15 min at 37°C. Both the monocyte and normal lymphocyte (nonadherent mononuclear cell) preparations were stained for α-naphthyl butyrate esterase (nonspecific esterase) in order to assess their purity (15). In addition, all cell populations were stained with Wright/Giemsa for morphologic analysis. Platelets were prepared by centrifuging anticoagulated (0.4% sodium citrate) whole blood for 10 min at 250 × *g*. The supernatant was collected and the platelets were pelleted by centrifugation at 400 × *g* for 6 min. RBC were prepared by centrifuging anticoagulated whole blood and removing the supernatant and buffy coat.

The human leukemia cell lines CCRF-CEM, Daudi, K562, HL-60, and U937 were maintained in RPMI 1640 with 10% fetal calf serum. CCRF-CEM, a lymphoblastoid cell line derived from a patient with T-cell acute lymphocytic leukemia (16), and Daudi, a Burkitt lymphoma cell line (17), were obtained from the American Type Culture Collection. K562, a myeloid blast cell line (18), was obtained from Bernice Schacter (University Hospitals of Cleveland). The HL-60 cell line, derived from a patient with acute promyelocytic leukemia (19), was a gift of Robert Gallo (Laboratory of Tumor Immunology, National Institutes of Health). The U937 cell line, derived from a patient with histiocytic lymphoma and exhibiting some functional and

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Abbreviations: RBC, erythrocytes; RIA, radioimmunoassay.

morphologic characteristics of macrophages (20), was a gift from Paul Guyre (Department of Physiology, Dartmouth Medical School). The myeloid blast lines KG-1 and KG-1a were the gift of P. Koeffler (Division of Hematology-Oncology, University of California, Los Angeles) and were grown in α medium (Flow Laboratories, McLean, VA) containing 20% fetal calf serum and gentamicin (21, 22).

Cells from patients with acute myelocytic leukemia, acute lymphocytic leukemia, or chronic lymphocytic leukemia were separated from blood by Ficoll/Hypaque gradient centrifugation and cryopreserved at −70°C prior to use. After thawing, the cells were assessed for viability by phase-contrast microscopy and acridine orange/ethidium bromide staining; they were used only if >95% were viable. Two acute myelocytic leukemia samples, AML-B and AML-C, were determined by morphological and histochemical staining criteria to be myelomonocytic leukemias (M4 in the French, American, and British classifications) (23). AML-A was a monocytic leukemia (M5) and AML-D was an undifferentiated myeloid leukemia (M1).

Binding and Cytotoxicity Assays. Binding of monoclonal antibodies to cells was measured by replicate radioimmunoassay (RIA) as described (12, 24). The ability of these monoclonal antibodies to mediate complement-dependent cytotoxicity of leukemia and normal cells was measured in a modified microcytotoxicity assay (12, 25).

Cytofluorographic Analysis. Cells were incubated at 10^7/ml in 300 μl of hybridoma culture supernatant for 30 min at 4°C. Supernatants from the parent myeloma line P3-X63Ag8 and from a hybridoma secreting an IgM antibody reactive with an irrelevant antigen were used as controls. The cells were washed twice in phosphate-buffered saline containing 0.1% bovine serum albumin and 0.05% sodium azide and incubated with 25 μl of a 1:10 dilution of affinity-purified fluorescein-conjugated $F(ab')_2$ goat anti-mouse immunoglobulin for 30 min at 4°C. They were then washed and resuspended in 0.3 ml of the buffered saline/albumin/azide. The Ortho (Westwood, MA) Cytofluorograph system 50H with multichannel distribution analyzer 2103 and Ortho 2150 computer systems was used to quantitate binding of the monoclonal antibodies to various cell populations.

RESULTS

Initial Characterization of Myeloid-Specific Monoclonal Antibodies PMN 6, PMN 29, and AML-2-23. The hybridomas producing these antibodies were cloned twice by limiting dilution. The binding titer of the spent culture supernatant, defined as the dilution that gave 50% of maximal binding to neutrophils in a RIA, was 1:64 for PMN 6, 1:32 for PMN 29, and 1:1024 for AML-2-23. PMN 6 and PMN 29 are IgM antibodies and AML-2-23 is an IgG of the γ1 subclass.

The reactivity of these antibodies with normal peripheral blood cells as measured by radio-binding assay is depicted in Table 1. The antigens to which PMN 6 and PMN 29 bind are expressed only on neutrophils (not on monocytes, lymphocytes, RBC, or platelets). The AML-2-23 antigen is found on monocytes and neutrophils but not on RBC or platelets. Cultured monocytes that differentiated into macrophages continued to express the AML-2-23 antigen to the same extent at 4 weeks in culture. The low binding to lymphocytes was due to monocyte contamination because AML-2-23-positive nonadherent cells were positive for nonspecific esterase. Although the neutrophils of each individual tested expressed these three antigens, a marked quantitative difference in expression of each antigen was found among individuals. Similarly, the expression of AML-2-23 antigen on monocytes showed wide variation among individuals.

Table 1. Binding of monoclonal antibodies to normal peripheral blood cells determined by radioimmunoassay

Cell type	Binding, cpm/10^5 cells		
	PMN 6	PMN 29	AML-2-23
Neutrophils	1,200 ± 390 (640–1,550)	3,740 ± 470 (2,970–4,220)	550 ± 200 (440–880)
Monocytes*	0	0	1,290 ± 370 (830–1,820)
Lymphocytes†	0	0	100 ± 7 (90–110)
RBC	0	0	0
Platelets	0	0	0

Numbers shown are means ± SD of counts bound to 10^5 cells from five normal individuals; numbers in parentheses are ranges. The counts (250–300 cpm) bound by control hybridoma supernatants were subtracted.

* Prepared by adherence to plastic; 85–90% of adherent cells were positive for nonspecific esterase and were morphologically consistent with monocytes.

† Preparations contained up to 12% cells staining positively for nonspecific esterase. Nonadherent mononuclear cells positive for AML-2-23 by indirect immunofluorescence were isolated by using the cell sorter and were determined to be 100% monocytes based on nonspecific esterase staining.

Cytofluorographic Analysis. Fluorescence staining analyzed by cytofluorography indicated that 68–85% of monocytes and 72–91% of neutrophils bore the antigen recognized by monoclonal antibody AML-2-23 (Table 2). The small percentage of cells staining with AML-2-23 in the lymphocyte (nonadherent mononuclear cell) fraction were isolated by fluorescent cell sorting and found to be 100% monocytes by nonspecific esterase staining (data not shown). The fluorescence intensity profile of this antibody on monocytes (Fig. 1*A*) indicated that the majority of cells showed very intense staining. AML-2-23 showed a different fluorescence profile on neutrophils (Fig. 1*B*) and did not stain any neutrophils as intensely as it did monocytes.

The antigen recognized by monoclonal antibody PMN 29 was expressed on >95% of neutrophils but not on lymphocytes and monocytes (Tables 1 and 2). As indicated by RIA (Table 1), PMN 6 reacted only with neutrophils and not with lymphocytes or monocytes. Moreover, its binding was limited to 58–95% of neutrophils in different individuals (Table 2). Neutrophils stained with PMN 29 showed a wide range of fluorescence intensities (Fig. 2*A*). The majority of neutrophils stained with PMN 6 occurred in the lower fluorescence range and yet the

Table 2. Reactivity of monoclonal antibodies with normal peripheral blood cells determined by flow cytometry

Cell population	% cells stained		
	PMN 6	PMN 29	AML-2-23
Neutrophils			
Donor 1	95	96	72
Donor 2	60	95	75
Donor 3	58	96	75
Donor 4	75	95	91
Donor 5	93	99	85
Monocytes*			
Donor 1	0	0	82
Donor 2	0	0	68
Donor 3	0	0	84
Donor 4	0	0	85

Results are expressed as the percentage of cells staining, correct for background, by indirect immunofluorescence.

* See * in Table 1.

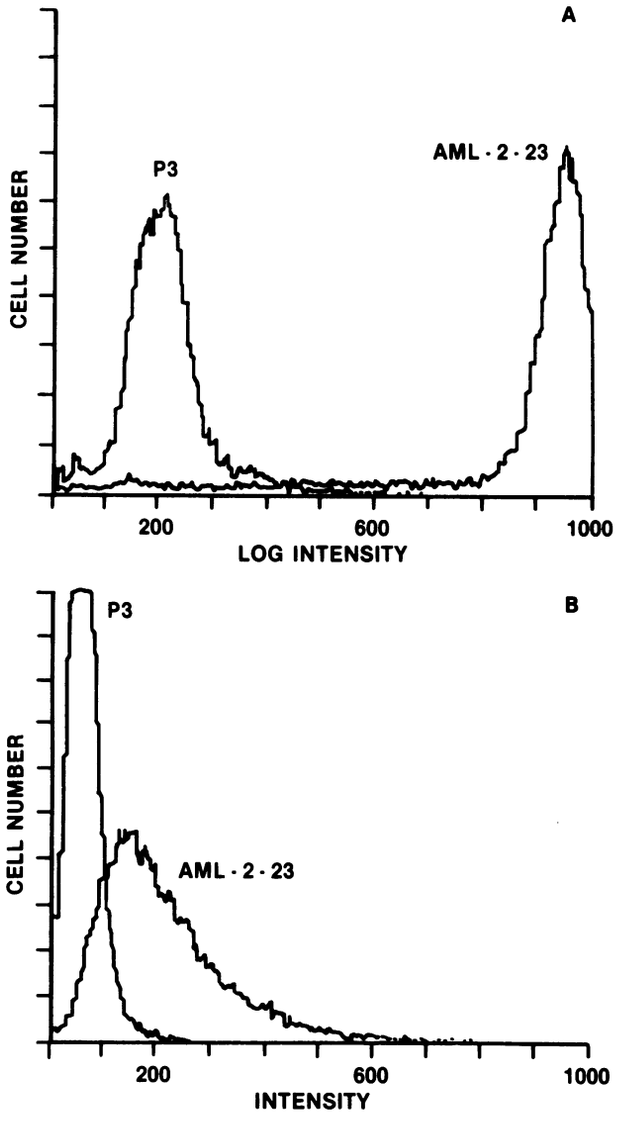

FIG. 1. Reactivity of monoclonal antibody AML-2-23 to monocytes (*A*) and neutrophils (*B*) revealed by indirect immunofluorescence and cytofluorography. Patterns of staining are shown relative to a control sample (P3) treated with supernatant containing IgG1 from the P3-X63Ag8 myeloma.

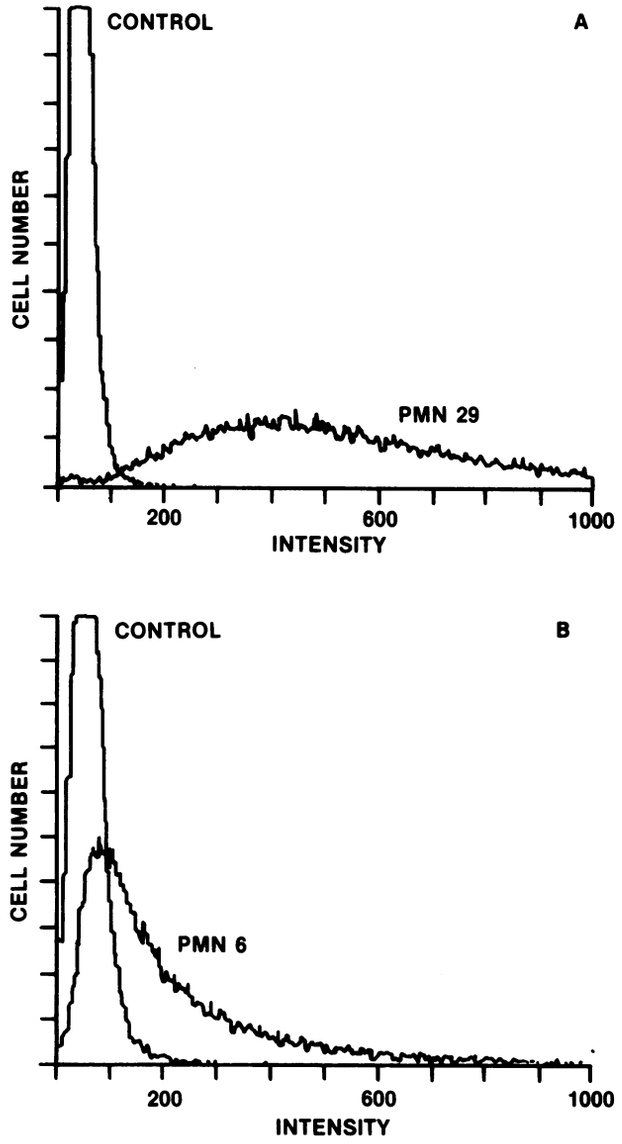

FIG. 2. Indirect immunofluorescence staining of neutrophils by monoclonal antibodies PMN 29 (*A*) and PMN 6 (*B*), studied by cytofluorography. Staining patterns are shown relative to a control sample treated with a monoclonal IgM of irrelevant specificity.

total range of fluorescence intensities observed with PMN 6 was also very broad.

Mean intensity of fluorescence staining was examined for comparison of average quantity of antigen on cells (Table 3). The antigen detected by AML-2-23 was expressed in far greater amounts on monocytes than on neutrophils from the same individual. Neutrophils from different donors also expressed varying mean amounts of this antigen. This variation was also observed with staining by antibody PMN 6 but not with PMN 29.

Cells from the lymphoblastoid lines CCRF-CEM and Daudi did not stain with either antibody (Table 4). A large percentage of cells of the HL-60 line, however, were positive for PMN 6 and PMN 29 and had mean fluorescence intensities similar to those obtained with positively stained neutrophils. A smaller percentage of cells also stained with AML-2-23 but with a mean fluorescence intensity greater than that of AML-2-23-positive neutrophils. Only PMN 29 stained the U937, K562, and KG-1 lines.

Complement-Dependent Cytotoxicity. Monoclonal antibodies PMN 6 and PMN 29 efficiently mediated complement-dependent cytotoxicity of neutrophils, selected leukemia cell samples, and the HL-60 cell line (Tables 5 and 6). PMN 6 was consistently cytotoxic to 30–75% of neutrophils from different individuals whereas PMN 29 was cytotoxic to virtually 100% of neutrophils from all individuals tested. AML-2-23 mediated cytotoxicity against normal monocytes, leukemia samples with myelomonocytic or monocytic morphology (12), and the HL-60 cell line to a titer of 2.5×10^{-4}. However, despite binding to neutrophils, AML-2-23 did not mediate cytotoxicity of these cells. PMN 6 and PMN 29 were also cytotoxic to myelomonocytic leukemia samples. None of these antibodies reacted with lymphocytic leukemia cells.

DISCUSSION

In this report we describe the properties of three monoclonal antibodies specific to myeloid cells. Two are specific for neutrophils, PMN 6 and PMN 29; the third, AML-2-23, is specific

Table 3. Degree of binding of monoclonal antibodies to neutrophils and monocytes as determined by flow cytometry

Cell type	Mean fluorescence intensity		
	PMN 6	PMN 29	AML-2-23
Neutrophils			
Donor 1	370	710	180
Donor 2	160	620	150
Donor 3	100	550	95
Donor 4	190	620	205
Donor 5	150	490	50
Monocytes			
Donor 1	0	0	>1,000
Donor 2	0	0	>1,000
Donor 3	0	0	>1,000
Donor 4	0	0	>1,000

Mean fluorescence intensity was calculated as follows:

$$\text{mean} = \frac{1}{N} \Sigma (N_x \cdot x)$$

in which N = total number of cells; N_x = number of cells in channel x; x = channel number. Results are expressed as mean fluorescence of the experimental sample minus the mean fluorescence of the control.

for an antigen present on both neutrophils and monocytes. All are unreactive with lymphocytes, RBC, and platelets. More detailed analysis by cytofluorography indicates that PMN 29 binds to virtually all neutrophils but PMN 6 and AML-2-23 react with a subpopulation of these cells. AML-2-23 also binds to 68–85% of normal human monocytes. None of these antibodies reacts with previously described neutrophil alloantigens (26) because all individuals studied expressed each antigen.

Two other human neutrophil-specific monoclonal antibodies have been reported to date. One identifies an antigen (My-1) present on neutrophils, bone marrow neutrophil precursors, and blast cells of some patients with myeloid leukemia (10). The other monoclonal antibody (NCD 1) was found to inhibit neutrophil chemotaxis and degranulation (4). NCD 3, a monoclonal antibody of undefined specificity, has been reported to inhibit *N*-formylmethionylleucylphenylalanine-induced chemotaxis of neutrophils (11). Like PMN 29, all of these antibodies bind to >95% of neutrophils. Although certain differences in the binding patterns of these antibodies exist, insufficient data are available to permit definitive comparison of their reactivities.

Monoclonal antibody AML-2-23 recognizes a determinant shared by both monocytes and neutrophils, as do a number of other monoclonal antibodies (4, 7–9, 27, 28). In addition, some hybridoma-derived antibodies that react only with monocytes

Table 4. Binding of monoclonal antibodies to cell lines determined by indirect immunofluorescence and flow cytometry

Cell line	% cells stained		
	PMN 6	PMN 29	AML-2-23
CCRF-CEM	0	0	0
Daudi	0	0	0
HL-60	57	80	14
U937	0	12	0
K562	0	11	0
KG-1a	0	0	0
KG-1	0	18	0

Results are expressed as the percentage of cells stained by monoclonal antibodies over background staining with an irrelevant antibody of the same immunoglobulin class and subclass.

Table 5. Complement-dependent cytotoxicity of normal cell populations mediated by monoclonal antibodies

Cell population	% cytotoxicity		
	PMN 6	PMN 29	AML-2-23
Neutrophils			
Donor 1	50–75	>90	0
Donor 2	30–50	>90	0
Donor 3	30–50	>90	0
Donor 4	30–50	>90	0
Donor 5	30–50	>90	0
Monocytes			
Donor 1	0	0	>90
Donor 2	0	0	>90
Donor 3	0	0	>90
Donor 4	0	0	>90
Donor 5	0	0	50–75

Cytotoxicity was estimated by dye exclusion in microtiter wells. Lymphocytes were not killed by any of these antibodies.

have been described (9, 29, 30). Most of these antibodies react with the majority of myelomonocytic and monocytic leukemia cells and some myelocytic blasts. One antibody, MY8, prepared by Griffin *et al.* (7) reacts with about 78% of adherent peripheral blood mononuclear cells and 72% of neutrophils. Although this resembles the reactivity of AML-2-23, some differences exist. MY8 binds strongly to the U937 and HL-60 cell lines whereas AML-2-23 only reacts significantly with the HL-60 cell line. This suggests recognition of different antigens, but definitive proof requires characterization of both the MY8 and AML-2-23 antigens. Sufficient qualitative and quantitative differences in reactivity also exist between AML-2-23 and other monoclonal antibodies to suggest that AML-2-23 binds to a determinant not previously recognized.

The fluorescence intensity profile of the AML-2-23 antigen on monocytes and neutrophils indicates a major difference between the two cell types in the expression of this antigen. Large amounts of the AML-2-23 antigen appear to be present on the majority of monocytes although some monocytes express little of this antigen. Neutrophils also are not homogeneous in their display of the AML-2-23 antigen. Similarly, the antigen defined by PMN 6 is not detectable on up to 40% of neutrophils but is present in high quantity on the others. Antibodies AML-2-23 and PMN 6 thus detect significant differences in cell surface antigen display among neutrophils which may reflect matura-

Table 6. Complement-dependent cytotoxicity of leukemia cells and cell lines mediated by monoclonal antibodies

Cell population*	% cytotoxicity		
	PMN 6	PMN 29	AML-2-23
HL-60*	50–75	>90	15–30
AML-A	ND	ND	>90
B	>90	>90	>90
C	>90	>90	>90
D	0	0	0
ALL-A	0	0	0
B	0	0	0
C	0	0	0
CLL-A	0	0	0
B	0	0	0
Hairy cell leukemia	0	0	0

Cytotoxicity was estimated by dye exclusion in microtiter wells. ND, not done.

* None of these monoclonal antibodies mediated cytotoxicity of the KG-1a, CCRF-CEM, Daudi, K562, and U937 cell lines.

tion in the circulation or functional diversity or both. The amount of antigen PMN 29 varied widely among neutrophils yet was expressed on these cells in greater quantity than the antigens bound by AML-2-23 and PMN 6. Based upon studies of myeloid cell lines, normal neutrophils, and monocytes, each of the three antigens appears to have a characteristic quantitative and qualitative distribution on myeloid cells at various stages of maturation. Some immature blasts of the KG-1a, KG-1, and K562 cell lines only express the PMN 29 antigen whereas the more differentiated cells of the HL-60 line display all three antigens to some degree. In contrast to the HL-60 line, a larger percentage of mature normal neutrophils express the PMN 29 and AML-2-23 antigens; the number of PMN 6-positive cells is the same in both cases. Studies on differentiation of the HL-60 cell line with dimethyl sulfoxide and phorbol esters (31) may provide more insight into the relative expression of these antigens on myeloid precursors at distinct stages of morphologic development.

The selective reactivity and cytotoxicity of these monoclonal antibodies to many myeloid leukemia cell samples may prove to be important in the identification and subgrouping of myeloid leukemia cells. Based on these and other studies (12, 24), it would appear that blast cells from many patients with acute myelocytic leukemia bear at least two of these myeloid antigens whereas all lymphocytic leukemia samples studied have been negative. PMN 6 and PMN 29 were found to mediate complement-dependent cytotoxicity in two cases of acute myelocytic leukemia studied whereas AML-2-23 selectively mediated lysis in six of six acute myelomonocytic and acute monocytic leukemia cell samples (12) but not in an undifferentiated myelogenous leukemia (AML-D). Thus, all three of these antibodies show considerable promise for both the diagnosis and treatment of leukemia. Furthermore, these monoclonal antibodies have considerable potential for investigations of neutrophils in disease states. Patients with congenital diseases in which disorders of normal neutrophil function are manifest (chronic granulomatous disease and Chediak–Higashi syndrome) or diseases in which there is a marked propensity for infection (diabetes mellitus or sickle cell anemia), for example, may have altered proportions of neutrophil subpopulations that reflect their functional capacities.

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